Effect of pH and Sodium Chloride on Growth of *Bacillus cereus* in Laboratory Media and Certain Foods

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The effects of NaCl concentration, pH, and water activity (a_w) on the ability of vegetative cells of *Bacillus cereus* to initiate aerobic growth in brain heart infusion broth at 30 C were studied in a factorial design experiment. By using multiple regression techniques, equations were derived which related the decimal reduction of the bacterial population to the concentration of NaCl and pH of broth to which the population was exposed. From these equations, the percentage of inoculated cells capable of initiating growth could be calculated. The reliability of these equations in foods was tested in laboratory-processed meat and rice media. The foods were less inhibitory than the broths, so that accurate prediction of growth initiation in foods was not possible by using the developed formulas. The impact of this type of quantitative study on the development of specific microbial standards for foods is discussed. When the NaCl concentration is increased, the a_w is decreased and, with increased deviation of pH from optimum, more concentrated inoculum of *B. cereus* cells is needed to assure initiation of growth in culture media and foods.

Bacillus cereus is a sporeforming bacterium which is widely distributed in nature. It has been recognized as a causative agent of food poisoning for almost 25 years. The symptoms are relatively mild and the duration is short. All kinds of foods can serve as vehicles, but typical ones are meat and certain meat products, as well as different types of puddings (10). Most epidemics reported have happened in Europe. In the United States *B. cereus* is rarely reported as the cause of foodborne disease: between the years 1968 and 1973, only seven outbreaks were reported (23).

It has been shown recently that one or more substances produced during the exponential and late exponential phases of growth can induce fluid accumulation in rabbit ileal loop (21), a necrotic reaction in guinea pig skin (8), and an altered vascular permeability in rabbit skin (9). These substances are produced also by strains of *B. thuringiensis* and *B. mycoides* (9), and they are different from lecithinase C and hemolysin and yet indistinguishable from *B. cereus* lethal toxin (8). Whether these substances are responsible for human food poisoning still has to be proven.

To evaluate the microbiological safety of a particular food, it is very important to know the percentage of inoculated cells capable of initiating demonstrable growth in that food environment. By using different preservation methods, this fraction of cells can be decreased to maintain a certain standard limit, allowing us to consider the food environment as safe. The effect of heat treatment on decreasing bacterial populations has been studied extensively. On the basis of studies done mainly with *Clostridium botulinum* spores, the canning industry has adopted the 12-D concept for low acid foods (16). This $1/10^{12}$ probability of a spore surviving canning is considered as a minimal safety standard.

Decimal reduction values for the effects of other preservation methods, except heating and radiation, are not generally found or used. Genigeorgis et al. (5) explored the possibility of applying derived equations to predict the probability that one staphylococcal cell will initiate growth in media at various pH values and NaCl concentrations.

Information concerning the effects of NaCl concentration and pH on the growth of *B. cereus* is limited (10, 22). The purpose of this study is to obtain additional information of the effect of these parameters on the aerobic growth of *B. cereus* in laboratory media. Equations were

developed which predict the decimal reduction of populations of various *B. cereus* strains exposed to such laboratory media. Practical applications of the equations were tested eventually in processed meat and rice environments.

MATERIALS AND METHODS

Preparation of experimental broths. The broths based on brain heart infusion (BHI) broth (Difco) were prepared as described previously (5).

Inoculation and incubation of broths. The following five *B. cereus* strains were used: ATCC 9139, ATCC 14579 and 2006 obtained from G. York, Department of Food Science and Technology, University of California, Davis, and 01552 and 5063 obtained from R. Wood and T. Midura of the California State Department of Public Health, Berkeley. The latter two strains had been isolated from food poisoning outbreaks.

Lyophilized stock cultures of the test organisms on porcelain beads were prepared according to a slight modification of the method of Hunt et al. (11).

In the experiments, overnight BHI broth cultures of the strains were inoculated into 25 ml of BHI broth containing 0.25% Tween 80 (Difco). The fresh cultures were incubated at 30 C on a reciprocal shaker for 4 h. The cultures were then centrifuged, the cells were washed once with saline containing 0.5% peptone (Difco), and the concentration of cells was adjusted to an optical density of 1.4 to 1.7 at 615 nm with a Spectronic-20 colorimeter (Bausch & Lomb). Nine tubes, each containing 9 ml of broth, were prepared from each type of experimental broth. A 1-ml amount of the cell suspension was added to the first tube, and nine 10-fold serial dilutions of the suspensions were prepared.

Three portions of 2 ml each were transferred with a sterile syringe from each of the nine tubes to 2-ml screw-cap vials. The caps were put on loosely and the vials were placed in 3-lb (ca. 1.4-kg) coffee cans (15.5-cm diameter by 17-cm height). In each can a vessel containing an NaCl solution of the same strength as the broth was placed. The cans were closed with their plastic lids, and the broths were incubated at 30 C for 10 days. Every other day vials with growth (turbidity) were removed and recorded. From the presence or absence of growth in the 27 vials prepared for each NaCl-pH combination, the most probable number of cells which had initiated growth was calculated from the tables of Fisher and Yates (4). The number of *B. cereus* cells present in the cell suspension used as inoculum was determined by plating on BHI agar (Difco) in duplicate. This number was always between 1.5×10^8 and 1.8×10^9.

Statistical methods. The experiments, arranged in a factorial design (20), involved five *B. cereus* strains, four NaCl concentrations (0, 2.5, 5, and 7.5%), and four pH values (4.6, 6.1, 7.5, and 8.8). For the statistical evaluation of the effects of NaCl and pH and their interaction upon the growth of *B. cereus*, the logarithm (log) of the ratio R_I-R_G was calculated for each broth and strain combination, where R_I was the number of cells in the inoculum and R_G the number

initiating growth. This log represented the number of decimal reductions of a bacterial population resulting from its exposure to a particular environment. The logs determined in the factorial experiments were evaluated by using the biomedical computer program (2) for multiple regression analysis. Equations representing the trend surface were constructed for each individual strain and for pooled data. Each equation related the effects of NaCl and pH levels on log decrease for that strain.

Preparation of experimental foods. (i) Meat. Cooked meats with different pH values and brine concentrations were prepared as described previously (6) and kept in the refrigerator until use.

(ii) Rice. Commercial enriched long grain rice (Town House) was washed with water and cooked for 15 min (until all cooking water had evaporated). The rice was allowed to cool to room temperature, and then it was divided into two lots, to one of which 5% (wt/wt) NaCl was added. The two lots of rice were homogenized in mortars. After overnight refrigeration, both lots were again homogenized and 0.2% (wt/wt) glucono-delta-lactone was added to decrease pH of certain samples. The rice media were packed, pasteurized, and stored in a manner similar to that of the experimental meats (6).

Inoculation and incubation of experimental foods. Small food disks used as growth media in the experiments were made by means of 9-mm diameter sterile cork borers and were placed in a sterile standard plastic petri dish.

B. cereus cell suspensions were prepared in a similar way as in the broth trials. Nine 10-fold serial microbial dilutions in sterile 0.1% (wt/vol) peptone were prepared. Portions of 0.01 ml from each dilution were inoculated on three food disks with a sterile microsyringe. The petri dishes containing the disks were then placed in 3-lb coffee cans. In each can there was a vessel containing a brine of the same NaCl concentration as that of the food sample. Incubation was similar to the broths.

After the appropriate incubation time, impression smears from each inoculated disk and uninoculated control disks were prepared on standard microscopic slides. The smears were examined for *B. cereus* growth with a Carl Zeiss phase contrast microscope. The most probable number of cells initiating growth was calculated in a similar way as in the broth experiments.

Physicochemical analysis of the growth media. Water content, NaCl and brine concentration, and pH of the experimental media were determined by methods previously described (6).

Water activities (a_w) of the experimental broths as well as food samples were measured by a model 15-3001 electric hygrometer (Hydrodynamics, Inc., Silver Spring, Md.) equipped with a gray band hydrosensor (range a_w, 0.81 to 0.99). Recalibration of the sensors was based on the use of NaCl solutions of different known molalities and a_w values at 25 C (17). A 30-g sample of material was put into a 200-ml Kerr mason jar that was allowed to stand at 25 C for 24 h for humidity equilibrium before the dial reading was taken. Two unused sensors were utilized.

Water activities of the experimental broths were also determined by an equilibrium moisture absorption technique as described by Vos and Labuza (24), based on the use of avicel microcrystalline cellulose.

RESULTS

The raw data of the combined effects of pH and NaCl of the broths on the log (decimal) decrease of the populations of the five *B. cereus* strains exposed to various broth environments are presented in Fig. 1. From the multiple regression analysis of the data, the following five equations were derived for the strains used, based on the first four levels of NaCl: strain 5065, $Y_e = 40.49 - 0.87$ (salt) $- 10.44$ (pH) $+ 0.04$ (salt)$^2 + 0.66$ (pH)$^2 \times 0.22$ (salt $\times$ pH); strain ATCC 14579, $Y_e = 54.48 - 1.54$ (salt) $- 14.68$ (pH) $+ 0.10$ (salt)$^2 + 0.97$ (pH)$^2 + 0.24$ (salt $\times$ pH); strain 2006, $Y_e = 38.20 + 0.27$ (salt) $- 10.87$ (pH) $- 0.06$ (salt)$^2 + 0.76$ (pH)$^2 + 0.16$ (salt $\times$ pH); strain ATCC 9139, $Y_e = 39.37 - 0.54$ (salt) $- 10.32$ (pH) $- 0.003$ (salt)$^2 + 0.67$ (pH)$^2 + 0.21$ (salt $\times$ pH); strain 01552 Y_e $46.13 - 1.33$ (salt) $- 12.27$ (pH) $+ 0.07$ (salt)$^2 + 0.81$ (pH)$^2 + 0.23$ (salt $\times$ pH). When the data of all the strains were pooled, the summary equation was as follows: $Y_e = 44.26 - 0.80$ (salt) $- 11.88$ (pH) $+ 0.03$ (salt)$^2 + 0.79$ (pH)$^2 + 0.21$ (salt $\times$ pH). Statistical analysis of the data indicated significant effects of (salt), (pH), (pH)^2, and (salt $\times$ pH) upon the magnitude of log decrease. Although the effect of the term (salt)^2 on the log decrease was not statistically significant, it has been retained in the equations for symmetry.

By using the equations given above, response curves were constructed for each strain and for the pooled data relating pH, NaCl, and log
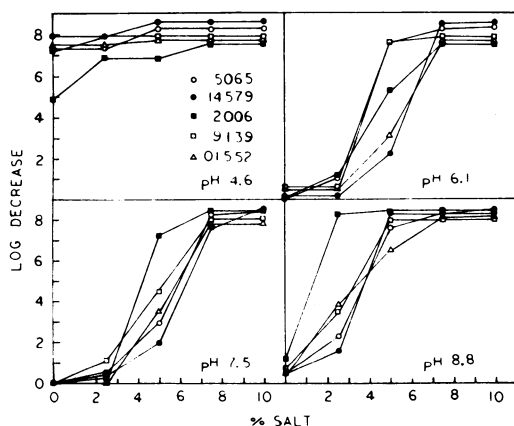
reductions. Curves for strains 20006, 01552, ATCC 14579, and pool data were chosen in Fig. 2. The standard errors for strains 5065, ATCC 14579, 2006, ATCC 9139, and 01552 as well as for the pooled data were 1.63, 1.66, 1.78, 1.60, 1.26, and 1.48, respectively. Approximate 68% confidence contours for a specified log reduction can be obtained by using $Y_e \pm$ standard error, and approximate 95% confidence contours by using $Y_e \pm 2$ standard errors. A response curve and the approximate 68 and 95% confidence limits for 4-log reduction of *B. cereus* strain ATCC 14579 are presented in Fig. 3.

To test the above-developed equations in foods, the log decreases of bacteria populations caused by meat and rice environments with different pH values and NaCl concentrations were determined for *B. cereus* strains 5065 and 01552 (Table 1).

The a_w of the BHI broths with 0, 2.5, 5.0, 7.5, and 10% brine were found to be 1.000, 0.985, 0.965, 0.955, and 0.935, respectively, as determined by electric hydrometer and 0.990,
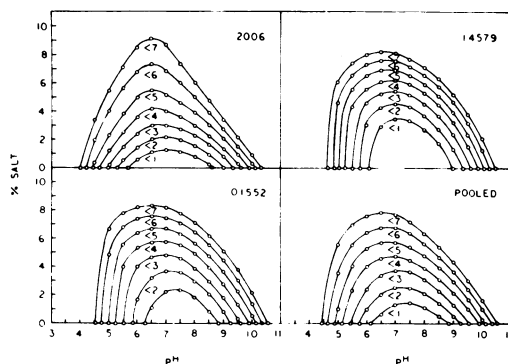
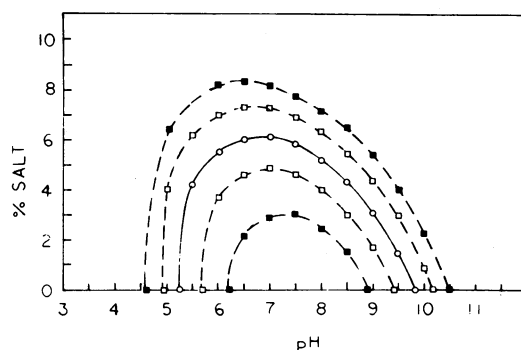FIG. 2. *Calculated log decreases for B. cereus strains studied.*

FIG. 1. *Effect of pH and NaCl concentration of BHI broth on the log decrease of populations of five strains of B. cereus.*

FIG. 3. *Response curve (O) and approximate 68 (□) and 95% (■) confidence limits for 4-log reduction of B. cereus strain ATCC 14579 exposed to different pH and NaCl combinations.*

TABLE 1. *Expected and observed log reduction of two food poisoning B. cereus strains exposed to rice and meat environments of different brine, pH, and a_w values*

Sample	Food	% NaCl in brine	pH	a_w	Log decrease[a]			
					Strain 01552		Strain 5064	
					Observed	Expected	Observed	Expected
1	Rice	0	0.75	1.000	0	0.21	0	0.99
2	Rice	0	5.00	1.000	1.02	5.03	3.24	4.79
3	Rice	7.5	6.80	0.965	>5.26	5.84	>6.48	6.96
4	Rice	7.9	5.15	0.960	>5.26	7.64	>6.48	8.80
5	Meat	0.0	4.35	1.000	4.43	8.08	5.76	7.55
6	Meat	5.6	4.35	0.975	>5.36	8.03	>6.00	9.30
7	Meat	10.1	4.35	0.935	5.36	11.89	>6.00	12.49
8	Meat	0.0	6.10	1.000	0.00	1.43	0.07	1.37
9	Meat	4.1	6.10	0.980	0.00	4.33	0.07	4.80
10	Meat	9.6	6.10	0.940	>5.36	8.58	>6.00	9.58
11	Meat	0.0	7.85	1.000	0.00	0.00	0.00	0.00
12	Meat	4.6	7.85	0.980	1.12	3.38	1.76	3.98
13	Meat	8.8	7.85	0.95	>5.36	9.32	>6.00	9.84

[a] Largest inoculum used: 1.8×10^5 cells/disk for strain 01552 and 3.0×10^6 cells/disk for strain 5065 in the case of rice and 2.3×10^5 cells/disk for strain 01552 and 10^6 cells/disk for strain 5065 in the case of meat. Food samples were incubated at 30 C for 10 days. To obtain 68 and 95% confidence limits for the expected log decrease, add or subtract 1 and 2 standard errors (SE) to the reported values. 1 SE = 1.26 for strain 01552 and 1.63 for strain 5064.

0.975, 0.960, 0.945, and 0.925 as determined by the microcrystalline cellulose method. No significant difference could be found between the results obtained by using the two different sensors in the electric hydrometer method.

DISCUSSION

The range of pH permitting growth of *B. cereus* in laboratory media has been reported to be pH 4.9 to 9.3 (10). The few articles (13–15) dealing with growth in foods of different acidity give the minimal pH for growth varying from pH 4.5 to 5.15. According to existing data (12, 19, 22) *B. cereus* is able to grow in 7% NaCl but not in 10%. Minimal a_w value allowing growth is reported to be 0.950 (18).

Previous data agree generally with the findings of the present study. Initiation of *B. cereus* growth in a meat environment at a pH as low as 4.35 has been observed for the first time. However, a high inoculum (10^6 cells in 0.01 ml) was used, thus giving greater probability of initiating growth. The maximal pH used in the study was 8.8. Though this pH was found to be relatively noninhibitory to growth of *B. cereus*, its significance to food protection is minimized by the fact that very few foods have such a high pH.

In this study the effects of NaCl and pH on the log reduction of *B. cereus* populations ex-

posed to different environments were studied. The quantitative approach used permits prediction of the percentage of inoculated cells capable of initiating growth, when inoculum and environmental parameters (% NaCl, pH) are known. This predictive potential is important in the development of microbiological standards for different types of foods. Equations were derived relating NaCl concentration and pH of BHI broth medium to log reduction of a *B. cereus* population exposed to this environment. The probability that one cell can initiate growth can be calculated from these equations. For example, for a broth at pH 5.5 with 2% NaCl, the log reduction for strain 5065 is 3.88, the antilog is 7585, and the probability of initiating growth is that 1:7585 or 0.0134% of inoculated cells will be capable of initiating growth. If salt concentration is increased to 4%, then 0.0011% of inoculated cells will be capable of initiating growth. Similar calculations can be done to any NaCl-pH combination.

Growth characteristics of *B. cereus* do not differ qualitatively from the general bacterial behavior pattern when exposed to different NaCl and pH conditions. These shapes of response curves resemble those found by Genigeorgis et al. (5) in their study on growth of *Staphylococcus aureus*. The general findings can be summarized as follows. (i) The effects of pH and NaCl on growth of *B. cereus* vary with strain and growth medium used. (ii) There is a

decreased rate of growth of *B. cereus* when exposed to media with NaCl concentrations increasing from 0 to 10%. (iii) When we increase the NaCl concentration of a medium, more concentrated inoculum is needed to assure initiation of growth. The same is true for pH when the values drift away from pH 6.5 to 7. (iv) High NaCl concentrations and extreme pH values prevent growth. (v) Smaller concentrations of NaCl are required to inhibit initiation of growth at pH values remote from optimum.

Food served as vehicles in epidemics of *B. cereus* food poisonings are usually highly contaminated. Microbiological examinations have regularly revealed contamination levels of 10^6 to 10^7 cells per g of food involved in food poisonings (10). The amount of contaminated food eaten by an individual before getting ill is, however, infrequently mentioned in reports concerning food poisoning epidemics caused by *B. cereus*. Generally, low attack rates for the sources of outbreaks as well as experimental feeding trials (10) indicate that a relatively large amount of a contaminated food must be consumed in order to produce symptoms. Because of the relatively low prevalence of reported food poisoning cases caused by *B. cereus* and the mild nature of the disease, no standards have generally been established for foods. To prevent *B. cereus* growth to as high levels as mentioned above, the growth environment should be inhibitory enough to reduce the probability of growth initiation at least by a factor of 10^4. The response curve for 4-log decrease for a population of *B. cereus* strain ATCC 14579 in BHI broth is presented in Fig. 3. Any NaCl-pH combination above the response curve causes the desired minimal inhibition. Figure 3 indicates also the approximate 68 and 95% confidence contours of the response curve. The curves are specific for this strain and growth environment and, as such, cannot be applied to foods.

To test the reliability of the formulas developed for BHI broths in food environments, experiments were made in which laboratory-processed meats and rices served as growth media. These types of foods have frequently been reported as vehicles in *B. cereus* food poisoning epidemics (10, 23). The data collected (Table 1) indicate that the food environments tested are remarkably less inhibitory than BHI broths. Thus, the equations will give too high log reduction values if applied directly in foods. For instance, strain 5065 inoculated into meat with 4.1% NaCl concentration in brine and pH 6.1 had a log decrease of 4.80 instead of 0.07 measured. Similarly, for the same strain, a

meat environment of 4.6% brine concentration and pH 7.85 would have a log decrease of 3.98 instead of 1.76 measured. Rice also appears to be a better growth medium than BHI broth. Sample number two (Table 1) had 0% salt and pH 5.0. The equations for strains 01552 and 5065 and such a salt-pH combination produced "expected" log decreases of 5.03 and 4.79, respectively, instead of 1.02 and 3.24 log decreases obtained experimentally in rice. Genigeorgis et al. (7) have also obtained less inhibition of staphylococci inoculated in processed meats than the predicted level of inhibition based on formulas derived from studies on BHI broths. To make accurate predictions of the probability of growth initiation in a certain food, the formulas should be developed for that particular food item as bacterial growth media. Glucono-delta-lactone was used as an acidulant for the food samples and HCl was used for the broths. However, this fact cannot explain the differences between inhibitory capabilities of the environments. At least in the case of *Salmonellae*, HCl has been shown to permit growth at lower pH values than gluconic acid (1).

Foodborne bacterial pathogens in general grow at a_w levels of 0.83 to 0.999 (22). In this high range, measurements by the electric hygrometer having a moisture-sensitive, salt-coated probe are considered to be inaccurate, especially when the probe gets older (3). In these experiments two sensors were used, one new and the other several years old but unused. When calibrated before use, no significant differences could be found between the results. Shape of the standard curves was, however, different. The new sensor gave remarkably vaster ranges for dial readings at a_w values over 0.9, thus giving better accuracy. New hygrometer probes are considered to be accurate within $\pm$ 0.005 a_w inside their specific ranges, that in this case (a gray band sensor) ends when a_w = 0.99. Water activities greater than that were reported as a_w = 1.000.

The water activities measured by equilibrium moisture absorption of the microcrystalline cellulose method were generally 0.01 a_w units lower than those given by electrical hygrometer. This trend does not agree with the results of Vos and Labuza (24), who got about equal values by both methods.

The lowest limit of a_w = 0.950 for growth of a vegetative *B. cereus* cell has been stated (18). The results of this study agree with the reported information. The lowest a_w value that permitted growth was 0.955 when measured by an electric hygrometer.

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